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CONCERNING THE INFLUENCE OF POST-MATURITY OF OVA ON THE SEX RATIO OF FROGS AND BUTTERFLIES

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CONCERNING THE INFLUENCE OF POST-MATURITY
OF OVA ON THE SEX RATIO OF FROGS AND BUTTERFLIES

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ABSTRACT. The author investigates the causes underlying the sharp sexual redistribution of frogs produced from overripe eggs. The changes result solely from alterations to the ova. The author finds that: overmaturity accelerates the differentiation of the testes; the author's earlier conclusion that changes were produced in the course of egg maturation, was incorrect. In the case of butterflies, overmaturity has no physical effect on the male sex, as it does in frogs. Overmaturity produces rather an increase in the number of females.

In earlier investigations done in part by me and in part by my students /269* Kuschakewitsch and Witschi which I summarized in a paper in the Biologisches Zentralblatt, it was shown that very overripe eggs in frogs result exclusively in male offspring, while in cultures of normally ripe eggs, equal numbers of males and females appear. The experiments were organized in the following way: To obtain normal sex distribution, a pair of animals was permitted to give up that part of their eggs which was used for further cultivation. Then males and females were separated and kept in cool storage. After an interval of 60 to 90 hours, the remainder still in the uterus was fertilized artificially. In this way, the previously-mentioned complete alteration of the sex distribution was achieved.

Now it is relevant to discover the causes which underlie this remarkable phenomenon. I was able to show in earlier investigations that changes in spermatozoa are not relevant to the question; I had frogs sent to me from the region around Florence where the germination time of Rana esculenta, with which I am working, appears several weeks earlier than in Munich. Furthermore, I obtained frogs from the region around Munich in areas where spawning had already /270 begun and also from areas where it had not begun and thus I was able to use sperm of variable ages in the artificial fertilization of identically-ripe ova, without finding any alteration in the sex proportion in the course of these experiments. Therefore any changes must reside solely in the ova. This was already apparent just from general considerations. Spermatozoa at the time of fertilization are cells which have completed their developmental process in toto; they consist chiefly of nuclear material and contain only a few mitochondria and practically no protoplasm. Therefore, significant changes in their behavior are almost entirely ruled out. This would suggest a change in the nucleus and protoplasm, for which the preconditions do not exist. How different for the ova cells! They have a rich supply of protoplasm and yolk material, their maturation is incomplete, but rather they remain at the time of overmaturity at the stage of the second metaphase. Thus they offer most favorable

*Numbers in the margin indicate pagination in the foreign text.

preconditions for alterations. The fact that significant changes occur as a result of over-maturity can also be derived from the observation that eggs which remain in the uterus for a long time, after several days -- the time is dependent on the external temperature -- lose their viability, which is not true for the spermatozoa. At least the experiences described above militate against that.

Changes in the ovum can occur in the stages of progamete and of metagamete, or can occur before or after fertilization. First, we shall discuss the possibility of changes in the progamete stage which I discussed in great detail in my previous publication. At that time as well as under the present circumstances relating to the sexuality problem, it was relevant to think about the influence of the maturation process. At one stage, the latter is being made in its course toward maturity. In amphibians the ova remain at the stage /271 of the second metaphase as long as they are in the uterus. The second polar body formation is formed only as the ova enter the water and indeed in a short time, if fertilization takes place, and much delayed, if fertilization does not take place. Moreover, we know from investigations on sex-determining chromosomes that the maturity of the sexual cells represents a critical stage, that generally it is decisive for the sex. The usual situation is that sexual determination derives from the masculine sex and is determined in the course of maturation of the spermatozoa, in that two kinds of spermatozoa are formed, those resulting in female offspring with X chromosomes and those resulting in male offspring without the X chromosome. Thus, the male is heterogametic, the female must in contrast be homogametic, since all eggs must contain the X chromosome. We are aware, however, of cases in which the inverse relationship obtains, i. e., the female is digametic and produces two kinds of eggs, with and without sex-determining chromosomes, which are designated for purposes of discussion as the Y chromosome. The male will, in contrast, be homogametic and produce only spermatozoa all of which contain the Y chromosome. Ten years ago the only case of a female d i g a m e t e was the egg of the sea urchin; however, it turned out that this was in error. In contrast, it has been shown in the meantime without any doubt that butterflies do have digametic ova.

Unfortunately at this time we do not have any definite information concerning the sex-determining chromosome in amphibians. The few investigations which have been published until now, claim digametic spermatozoa; they have had no general recognition. I performed no further pertinent investigations. My results, which derive from microscopic sexual examination of my test materials (exclusively young frogs and frog larvae) have given me no reason to form the assumption of sex-determining chromosomes, either for masculine or feminine frogs.

If in fact the spermatozoa are digametic, then there are relationships /272 which would be difficult to clarify. In this case, under normal conditions all mature eggs would contain the X chromosome, while the same would be true for only half of the spermatozoa. If the highly unlikely hypothesis that those spermatozoa which give rise to female offspring, the spermatozoa with the X chromosome, are excluded from fertilization, is to be avoided, then one must assume that those eggs whose polar body formations occur under the conditions of overmaturity, (which when fertilized with spermatozoa containing X chromosomes, which would normally result in female offspring,) produce instead male offspring. Since fertilized eggs give rise to male offspring only if they contain one X chromosome, then eggs which are fertilized with X spermatozoa cannot themselves contain an X chromosome. All eggs must have lost their X chromosome which they acquired in the normal process of maturation. From this, a new difficulty arises. For if such ova are fertilized with spermatozoa without an X chromosome which would normally result in a male offspring, then such male offspring would arise which would contain no X chromosome in the diploid nucleus and therefore would only themselves give rise to spermatozoa of one kind. These would not offer the possibility of female offspring when used with normally mature eggs, but rather only normal male offspring with an X chromosome which derives from the ovum. The male offspring which were the result of overmaturity would consist of two types: The one half containing normal chromosomal constitution, the other half not, a difference which would appear in the production of the next generation.

Digametic ova would permit a simple explanation of the results of over-maturity. In such a case, the diploid nucleus of the male sex would have to contain two Y chromosomes, the female only one Y chromosome. The distribution in mature eggs would result in this way in the situation that all eggs would retain the Y chromosome in the mature distribution. Since all spermatozoa are equipped with the Y chromosome, then eggs with two Y chromosomes could arise /273
in fertilization, i. e. male offspring.

I have previously declared myself in favor of this latter explanation. Goldschmidt (1921) and Seiler (1921) also agreed. The latter had in fact attempted to offer proof for this explanation in butterflies, for which he had shown the digametic state of the female, by fertilizing overmature butterfly ova and testing their maturity distribution as to chromosome composition. Here he used Psychide Talaeporia, whose eggs can be easily cut: they offer favorable conditions for the study of chromosomal composition. Among 247 eggs in which the cutting fortunately encountered the polar body formation, it could be determined that in fact the Y chromosome was retained in the egg during anaphase in 146 cases and in 101 cases migrated into the polar body. Assuming the validity of our assumption, the 146 eggs would be male and the 101 eggs female. That would yield a sex count of 144 (144 ♂ over 100 ♀), while under normal conditions it amounts to 74.

If the explanation of the effect of over-maturation is correct, then its validity could be tested by means of culture experiments. Experiments would only have to be performed in animals whose female gametes are digametic, ignoring all the rest of the cases in which the male gamete is digametic. Insects are very favorable subjects for such investigations, since in several orders (Orthoptera, Diptera, Rhynchota) male, in others (Lepidoptera), female digametes are prevalent. Therefore, I have begun to extend my investigations in this direction, which will be the subject of the following lecture.

I come now to the second possibility which *m a y e x p l a i n* the results of over-maturity, the possibility of metagametic influence. We must then assume that polar body formation and the distribution of chromosomes /274
which is tied up with it is not altered by over-maturity, but rather after fertilization, influences make themselves felt, which are caused by over-maturity and which alter the behavior of the normal chromosomal mechanism.

Such influences on the chromosomal mechanism can arise only from the protoplasm. Even earlier I found occasion to consider such influential factors since even then there were observations available that a female chromosomal composition can be converted into a male composition. An excellent example of this was recognized at that time, the hermaphroditism of Rhabdonema nigrovenosum. The animal resulting from the fertilization is a female with two X chromosomes; there is then the possibility of producing, besides eggs, spermatozoa as well in which, in a part of the sex cells, the second X chromosome is removed. Apparently other cases of hermaphroditism can be explained in similar fashion. A further example certified by observation of the alteration of the chromosomal mechanism by influences which apparently derive from the protoplasm, are the aphides among whom in the course of parthenogenetic development eggs which would normally produce females become thereby male eggs, which reform an X chromosome. A third example of metagametic sex-determining alteration of eggs has been made available by Baltzer, even if the behavior of the chromosomes in this case has not been certified. Baltzer investigated Bonellia viridis and found that the eggs of this animal develop into female offspring if the larvae are allowed to swim free in the water. If, however, the larvae are set tightly on the snout of the mother and thus are converted to a parasitic mode of living, then they produce males. If the parasitic life mode is interrupted too early, so that complete sexual conversion is not obtained, then hermaphrodites of various types arise.

Now it is by no means necessary in order to explain the sexual altera- /275
tion of eggs to assume that a second X chromosome must be reformed; it would be far more satisfactory to assume that a diminution of its sexual potential is brought about. We must figure on such a possibility thanks to the very meaningful results which Goldschmidt obtained in his investigation on sex determination in Lymantria dispar. In the case of this animal, there is a myriad of different races which are distributed over the various continents. Goldschmidt used for his investigation domestic and Japanese races. If males and females are chosen for experiments in sex determination, which belong to the same race, the normal sex ratio of 50 ♂:50 ♀ is obtained in every case. If on the other hand different races are crossed with one another, then deviations from the normal appear, which are more or less striking, according to the races which have been combined. In many combinations, the male offspring are greater in number, while in others the females outnumber the males. If the male component is preponderant, then 50% normal male offspring are obtained while among the 50% female offspring there are animals which are altered in the masculine direction. If the increase of male preponderance or, to use an expression of breeders, of the male penetrating power, is not very great, then only some of the secondary sex characteristics of the female, above all the color of the wings, are changed in the masculine direction. If the male preponderance is very much increased, then the ovaries are affected as well in that hermaphroditism occurs. Among the highest grades of preponderance, finally, the entire culture consists exclusively of males, of which about 50% show markings which allow recognition that they have been derived from female eggs. By means of appropriate selection of races, a spectrum of intersexual forms from pure female all the way to fertile males can be achieved. Likewise, it is pos- /276
sible to obtain a continuous distribution in the reverse direction, a distribution which goes from male to female.

In explaining his findings, Goldschmidt very understandably avoided the assumption of changes in the chromosomal apparatus, but rather sought to make the circumstances understandable in the fashion which was explained above, such that the male and female potentialities within a single race correspond correctly to each other, to achieve the normal sex distribution. He further stated that different races, however, behave differently from this standpoint so that in the case of the relatively increased male potential of one race the result of a cross would displace the distribution more toward the masculine side and vice versa. If we make use of these observations for the results in work with frogs then we could abandon the assumption of some kind of chromosomal reformation and satisfy ourselves with some kind of diminution of sexual potential, a diminution which in the case of frogs is brought about by overmaturity, something that occurs in the case of Lymantria through the choice of appropriate races.

Another point to be considered would be whether selection processes of a particular kind could have any effect on the alteration of the sex distribution. We know of cases in which only female offspring arise because the spermatozoa capable of producing males are infertile. Since, as pointed out earlier, changes in the spermatozoa cannot be considered, then in the case of frogs the only consideration would be that a counterpart would be the infertility of female eggs. This, however, is contradicted by the fact that in most over-mature cultures, all the eggs can be fertilized. Moreover, we know of cases, to be sure only in the plant kingdom, in which both ova and spermatozoa are viable and despite this a selective heteroization occurs because a kind of competition appears in male- and female-producing pollen whereby one can outflank the other in achieving the ova to be fertilized. Similar circumstances /277 are possible among plants where the pollen utricle must traverse a long distance from the hilum of the pistil to the fruit anlage or also among vertebrates with internal fertilization among whom the spermatozoa must traverse a long oviduct before they can reach the egg. However, the differences cannot be that large among the amphibians; in fact, they must be very negligible as viewed from the standpoint of the circumstances that in artificial fertilization every egg is in direct contact with hundreds of spermatozoa. A selective fertilization would therefore have to be noticeable in the case of over-mature ova.

Finally, there is one last possibility, the possibility of a selective development, such that all eggs indeed begin to develop, however the female eggs die off during development. We certainly know from the human example that the mortality of male zygotes is greater than that of female zygotes. In the case of frogs, it could be that the opposite occurs under conditions of overmaturity. Furthermore, we learned from the very elegant investigations of Morgan that the existence of sex-linked lethal factors, of factors which have an effect only in one sex and cause that sex to die off. This possibility is contradicted, however, by the fact that in many cultures it is well known that the eggs develop so far that the sex can be determined. In such case, it would be expected that there would be several female offspring among the prematurely-dying larvae, which is not the case. To be sure, the number of favorable cultures of this sort which have been achieved is very small.

The degree of mortality is usually directly proportional to the degree of over-maturity, so that many larvae die at a very early stage of development.

The discussion which I have given thus far allows the conclusion that it is necessary to continue the investigations which I began over a decade ago. This has been my intention for a long time. The intent to carry out this plan /278 has, however, long been delayed. At first because of personal reasons, later because of the war, which robbed me of the necessary assistants for these investigations. As somewhat normal conditions returned at the conclusion of the peace, I sought to take up my work again in the spring of 1919, however, I had to battle against great difficulties. The planned construction of a large glass house for aquaria and terraria which was approved before the war had to be abandoned because of the enormous costs which were due to the general price level increases. The renovation of the aquarium installations which were planned instead of the new construction was further delayed as a result of the confusion caused by the Weimar Republic, so that I had to continue the investigations which I had begun earlier under very unfavorable conditions. At that time I experienced the outbreak of an epidemic -- perhaps as a result of the unfavorable culture conditions -- which completely exterminated the larger part of my cultures, and decimated another part so much that only a very few individuals survived. These events offered another confirmation that over-mature cultures consist only of males, but could not contribute to the clarification of the question concerning the causes of this operation in sex determination. Conditions were so much more favorable in 1920 that in the following discussion I will deal exclusively with these results. That same summer I also began my investigations on the effect of overmaturity in butterflies, in which I used Lymantria dispar. First I will discuss the results of my frog cultures and then my experiences with the Lymantria material.

Culture Experiments with Rana esculenta

Temperatures in the summer of 1920 were such that unusually warm days began with the first of May, 1920. This led to an early beginning in the /279 spawning period of Rana esculenta, which I had seen only exceptionally before, so that I was able to begin my investigations in the first part of May. I took my material exclusively from two small ponds just below the Walchstadt Heights. Experience gained in the previous seasons was the decisive factor for selection of these trapping areas. I have already mentioned in earlier publications that investigations concerning sex determination in frogs are made significantly more difficult by the wide distribution of a condition of the gonads which I have characterized as a condition of indifference, and will so designate in this publication, which other investigators designate as hermaphroditism following the example of Pfluger. This phenomenon is described in detail by Witschi and Kuschakesitsch and illuminated by their illustrations. Therefore I can refer to their work and need only describe here that which is necessary for understanding the problem.

I speak of indifference when the gonads form a long thin tissue strip which stretches over a large part of the kidneys. In one culture studied, the length varied from 1.5 to 2.6 mm. The part is swollen like a string of pearls because there are rows of small fluid-filled cysts, in the secondary genital

cavities. They number between 5 and 14, roughly proportional to the length of the cord. The right gonad of the frog generally contains fewer cysts, corresponding to a shorter gonad. In contrast, if the gonads are sexually differentiated, then the sexes can be differentiated from one another with great clarity without the use of a magnifying glass. The testes are short compact oval bodies, which are restricted to the highest section of the kidneys; the ovaries in contrast form either a long cylindrical sausage-shaped cord or wide, long and thick, wrinkled discs, in which the ova are recognizable as small kernels. Indifferent gonads predominate in many of the areas where the frogs are found. When such animals cannot be differentiated, even among those which have undergone meta- /280
morphosis, even animals which survived the winter and grew as a result of abundant food supply in the following summer, male and female animals are indistinguishable. In other cases the sex can be determined in very young larvae, in which the hind legs are still short stumps.

While a high degree of indifference of the gonads predominates in most areas where frogs were taken in the area around Munich, to which I can testify on the basis of manifold investigations (partly from cultured material, partly from collections in nature), the Walchstadt material is sexually well differentiated and therefore is very appropriate for investigations of sex determination, at least with respect to the chief problem. Even here, there are exceptions. We will even see that one of these exceptions became of particular interest in my cultures.

It would be important to report the class of such variable behavior of the sex apparatus. Is it a question of inherited racial differences or one of modifications which are caused by external circumstances, temperature, nutrition and similar conditions? Goldschmidt surmised that temperature played a decisive role. He came to this conclusion on the basis of observations made by Pfluger with Rana temporaria. Pfluger had frogs shipped to him from various regions of Germany. It developed from this experiment that material coming from Konigsberg was sexually differentiated, while, in contrast, the material which was collected in Bonn and Luttich consisted mainly of "hermaphrodites." According to this, lower temperatures would favor sexual differentiation. Goldschmidt drew this conclusion as well. My own experiences make it clear that circumstances are not that simple. Very near the small ponds of Walchstadt are widespread peatbogs.
Sexual indifference was predominant among the frogs collected from them, and /281
similarly in the outlying peatbogs on the other bank of the Isar, furthermore in the region around the villages near Schleissheim and many other places. The peatbogs around Lochhausen in contrast yield material which is again favorable for investigation, i. e. with early sexual differentiation, while those from a none - too -distant village are characterized by indifference. Similar to findings in the Walchstadt ponds, there were many indifferent forms among the frogs in the Lochhausen area, even in rather significant numbers. All of this makes it apparent that temperature does not have any influence but rather that the differences are racial. This would be in agreement with the observation that the frogs from the Walchstadt and Lochhausen areas have similar somatotypes. They differentiate themselves from frogs from other areas in that they are smaller on the average. In any case, my experiences are not sufficient to decide the causes of sexual variability. Clarity on this issue will come only

after further detailed investigations, partly by collections throughout the year, partly by methodic cultures. And finally, I would hope to gain a decisive answer by "racial" classes.

The material which was used was collected in three expeditions, on May 8, May 13, and May 16. Of seven copulae [sic] sighted on May 8, four were captured; they were separated at the capture, reunited again after capture, only to be separated again very shortly. I obtained only one copula from the material, which remained united for a prolonged time from the 13th of May and after 24 hours, i. e., on May 14, finished spawning. I mention these circumstances as proof that a varying maturity of the eggs had not been reached on May 8. Problems occurred in the following time period in this investigation in that pairings occurred which lasted a short time, and then separated again. The most usable material was obtained on May 13, followed by that of May 16, so that the height of sexual maturity was reached at this time. Of those /282
copulae which were usable for the planned investigations, I used a total of seven for culture purposes; the others had to be abandoned as a result of the minute space available in the aquarium. Of these seven cultures, two were not usable at all in the overmaturity experiments, since the uterus was completely emptied with the first oviposition. I used them anyway at least to determine the sex ratios of normal ovipositions. Of the other five cultures, I left one untouched, since it was sexually indifferent to a high degree and therefore hardly usable due to the fact that the overmaturity of the artificially fertilized eggs which were used amounted to only about 48 hours and thus was so short that no real results could be obtained. Another pair came into question which derived from the second catch which remained in copula from the 14th of May until the 20th before it began to spawn. Thus there were four cultures remaining, the results from which I will report in the following. Three of them led only to a corroboration of results gained earlier; I can deal with them summarily, therefore. The fourth one, in contrast, demands separate and detailed discussion since it could lead to new points of view.

<u>Overmature Cultures 1-3.</u> Of the first fertilizations (normal fertilizations) I cultured only 100 eggs. I treated the other two cultures similarly, which were not appropriate for an overmaturity study, because the females had exhausted completely their supply of eggs. In all cases, early and clear differentiation between males and females was possible; the following list gives five sex determinations. In the three cultures in which an overmature culture was possible, I likewise present the sexual ratio, and simultaneously the time interval between the first and second fertilization.

	Normal Fertilization		Time Interval	Overmature Fertilization	/283
I.	49 ♂ 44 ♀	93%	--	--	
II.	49 ♂ 41 ♀	90%	--	--	
III.	38 ♂ 41 ♀	79%	77 hours	431 ♂ 1 ♀	
IV.	45 ♂ 47 ♀	92%	68 hours	202 ♂ 12 ♀	
V.	45 ♂ 4 N. 45 ♀	94%	72 hours	40 ♂	
Total	226 ♂ 4 N.218 ♀	90%		673 ♂ 13 ♀	

I have no remarks to make with respect to the culture results except for a few words on the observed mortality. In normal fertilizations, this is very small. In overmature cultures, in contrast, the mortality is very high, even in Culture III, the first overmature culture. The very high mortality rate can be explained by the fact that an enormous amount of egg material is available for overmature fertilization. Worthy of note is the fact that even at 68 hours of overmaturity, a not insignificant amount of females can still be shown.

Overmature Culture 4. Since previous cultures have yielded the result that even in the presence of relatively small degrees of overmaturity the development of capability of the eggs had suffered strikingly, an interval of 67 hours was chosen for this fourth culture. The pair in question had come together on the 18th of May and began to spawn at 2:00 on May 19. During the time from 2:00 to 5:00 several hundred eggs had been deposited, when they finally were separated. Since earlier experiments which attempted a second fertilization by means of a normal pairing had yielded unfavorable results, the artificial fertilization was immediately performed on May 22 at 9:00 a.m.

I. Normal Culture. A not insignificant percentage of the eggs did not develop, so that in the course of the fifth and sixth days, 58 (approximately 15%) of dead eggs were separated. The rest developed without problems. As in earlier cultures, 100 eggs were raised in a culture aquarium at higher temperatures as a control culture, the rest being held as a reserve culture at room temperature and with moderate nutrition, with, however, daily change of water. /284
The loss quotient was not determined for the reserve culture. Among the control culture (warm culture) 94 animals survived the investigation in toto; of these, 46 animals were sacrificed on July 7, since according to experience obtained in other cultures it could be expected that the sex could be easily determined by this time. This turned out, however, to be an error. The larvae which were to be used in this investigation all had well-developed hind legs, many had even developed forelegs as well. However, all had still the indifferent character in the sex glands, a more or less long-drawn-out pearl-string-like swollen cord about 1.5 to 2.6 mm long. I attempted to make a separation of the two sexes by measuring out under the magnifying glass the length of the sex glands in a large group of the animals on both sides, to determine if the material could be separated into two groups in this way, a group with longer gonads, which one could designate initially as ovaries, and a group with shorter sex cords which I could assume to be male; between the extremes in size, there was a continuous transition from the standpoint of both the actual length and the count of pearl-like swellings. Separation was further hindered by the fact that, in general, the sex glands of the left side are longer and have many more swellings than those of the right side. Microscopic investigation of sections also led to no results. I selected the longest and shortest sexual organs and sectioned both, but was unable to recognize any difference in histological appearance.

In these circumstances, I decided to cultivate the rest of the normal culture to the state of metamorphosis. The metamorphosed culture was sacrificed in small parts during the months of August and September; a smaller remainder of seven animals was preserved for wintering and fed until July 1921. /285

The animals which were sacrificed before the winter still showed no definite sexual appearance. In most differentiated forms, the gonads were cylindrical in form, studded with eggs, so that one could call them ovaries. However, there were also several cysts in the periphery, which arise from involution and resorption of eggs and as appear when ovary-like gonads are converted into testes. In other cases, the entire gonad even to the small-celled rest in the axis of the gland consists of such forms which arise from the involution of cysts derived from eggs. Between these two extremes, there were intermediate forms, in which either cysts derived from the degeneration of ova or undamaged ova primordia predominated. Apparently the material investigated was thus obtained in a stage of differentiation of the sex glands such that no clear differentiation could be made as to how many animals would have differentiated to females, and how many to males. The material which survived the winter corroborates this without doubt. In this group, there was one animal with testes and six animals with strikingly wrinkled ovaries of characteristic appearance.

II. Overmature Culture. I had available for the overmature culture a very rich source of eggs which had remained in the uterus. I decided therefore to use three different ways of fertilization. The first part was fertilized by the method which I have used since the beginning of my investigation: The eggs were placed in a thin layer on the slide and an aqueous solution rich in spermatozoa was sprayed between the individual eggs with a pipette. With the second part, the spermatozoa-containing fluid was very dilute. This was done to avoid polyspermia, since in cases of extreme overmaturity this presents a significant danger. A third portion with the eggs covering the slide in a thin layer was dipped in a diluted sperm solution. The second and third methods yielded no good result. Of 567 eggs, 485 remained unfertilized, while with my usual method /286 of fertilizing eggs, only 56 remained unfertilized and 445 eggs developed. Thus I was able to begin the culture with a total of 527 larvae of which about 40 were lost in an accident, about 20 died, so that the culture consisted of more than 500 animals at introduction into the culture aquarium. Of these, on July 7, thus approximately at the same time in which the first sacrifice was performed with the normal culture, 77 individuals were sacrificed, and the rest were allowed to continue, partly to metamorphosis, partly in nutrition culture. A smaller part (8 individuals) was preserved over the winter and further cultured in the summer of 1921.

Investigation of such a complete set of material led to very interesting results. Among the 77 polliwogs in the first test sacrifice of July 7, there were 39 males and 38 indifferent specimens. The last apparently corresponded to those individuals which, according to their original constitution, would have developed into females. For, not only among my cultures, but also among the material collected in the wild, I was often able to document that the males were already clearly differentiated while the females remained at the stage of indifference.

With due consideration to the fact that the polliwogs of the normal culture which were sacrificed at the same time were all indifferent and the sacrifice of the late fertilization likewise did not give rise to a completely flawless sex determination, I decided to undertake to continue the overmature material to the completion of metamorphosis, and in part even beyond this time in a nutrition culture and to sacrifice individuals from time to time. A smaller remainder was allowed to survive the winter and was sacrificed after rich nutrition in June of 1921. The results were the following:

Of those allowed to complete metamorphosis, in part well-fed and sacrificed in the fall of 1920, the young frogs consisted of 313 males and 45 N. These frogs were distributed among both fertilization A with diluted sperm, and B with concentrated sperm, in roughly equivalent fashion: A 64 ♂, 5 J; B 249 ♂, /287
40 J. Of the eight animals which survived the winter, seven had definite testes and one had developed indubitably into a multiply wrinkled ovary. While the first sacrifice yielded the normal test relationship of 50 ♂ and 50 ♀, if we count the indifferent forms as females, this changed in the course of the culture to 700 ♂ : 100 ♀, in which the females, however, are not fully developed. This latter, however, was the case in 1921. These results permit only one interpretation, that there were added to the 50% ♂, a figure which derives from the first series of sacrifices, more males which accumulated in the course of the culture, deriving from delayed transformation of indifferent forms. Also in agreement with this interpretation is the fact that among those males sacrificed in 1920 there were not a few forms in which the testes were in such a condition as to differentiate from indifferent primordial glands. Especially significant for this interpretation are those cases in which a clear testes had developed on the right side while on the left side the gonad again appeared to be a transition to indifferent. I had already become aware during my earlier investigations that the gonads on the right side almost always not only, as mentioned above, are somewhat smaller, but are also more advanced in development.

Turning now to an interpretation of the results given above, two things are clearly apparent.

1. Overmaturity accelerates the differentiation of testes. While in the normal culture it was not possible to differentiate between males and females even beyond the time of metamorphosis, in the case of overmaturity it is possible at an even earlier stage, at least for that half of the culture which shows typical testes. This corresponds to an experience, which I have made repeatedly, that in normally-raised frog material, half of the individuals /288
have testes already, while the other half, apparently the half which is to yield females, remains indifferent.

2. The results of the cultures discussed above furthermore refute the interpretation which I represented earlier concerning the effect of overmaturity: That it produces changes in the course of egg maturation, that females represent the heterogametic sex, that with egg maturation the sex chromosome gets into the polar body and therefore only such eggs remain which are without the Y chromosome, and thus are able to yield only males. If this interpretation were correct, then all eggs in the overripe culture would be determined as males at the very beginning, indeed, in such a clear way, which was not the case in our investigations. Quite the opposite. Even at a rather early stage, two kinds of larvae could be distinguished, of which one was indubitably male, the other, consisting of eggs which originally were destined to become female and only in the course of embryonic development, i. e. as metagamete, experienced

some kind of conversion. These circumstances are reminiscent of phenomena which we recognize in many hermaphroditic animals (Rhabdonema nigrovenosum), in which at first homogametic females arise, among which in the course of development the opportunity to produce testes is so brought about that in one part of the sex cells one of the two X chromosomes is lost. And so I would like to explain the situation this way as well. I assume that the male sex among frogs is the heterogamete as is generally claimed for frogs and other amphibia and for vertebrates in general, even on the basis of not completely flawless observations. Accordingly, two kinds of eggs must arise on fertilization, so-called male eggs with an X chromosome and female eggs with two X chromosomes. /289
The latter experienced in my experiment under the influence of overmaturity in the course of development a transformation, either because the second X chromosome completely involuted, or was weakened in its effect. I think it is apparent that the influences which cause the conversion of the chromosomal apparatus derived from the protoplasm, which is valid as well for hermaphrodites and not only for these, but also for animals such as daphnids, aphids, among whom in the course of their generation occurs likewise a sexual transformation, a complete transition from female to male sex. My feeling of the correctness of this representation is even more reinforced by my investigation concerning:

The Effect of Overmaturity on Butterflies

I was brought to these investigations by the briefly-discussed considerations above.

Through the elegant experiments of Seiler on the behavior of sex chromosomes in butterflies, it was shown, yet in this case in contrast to most animals studied thus far, that the female was a heterogamete, which was very apparent on the basis of Mendel's experiments already, and that the male sex is a homogamete. Among other insects, e. g. the recently well-studied Diptera, it is known to be the reverse. If my earlier interpretation of overmaturity experiments was correct, then it must be quite different with respect to butterflies and Diptera. Among butterflies, the same phenomena must appear as among frogs, while among Diptera in contrast, overmaturity evidently has no effect on sex.

From these considerations, I have recently begun to extend my overmaturity experiments to butterflies. I was supported very kindly by Professor Goldschmidt of the Kaiser Wilhelm Institute on Biology, who, among others, sent me on two occasions Lymantria dispar (Aomori and Gifu races), for which I am /290
very grateful to him. I would like to report my investigations only in summary fashion, since I see them now as only private experiments, which will be followed by extensive experiments only this year.

In butterflies, and probably also in all insects, there is a large disadvantage for overmaturity experiments in contrast to frogs, in that two clearly separable fertilizations cannot be temporally achieved, a normal fertilization and a late fertilization. One can proceed only in such fashion that butterflies which have recently become capable of breeding are allowed to pair in part immediately, and in part only after 2, 3, 4, etc. days, and draw a mean from a great number of cultures, to rule out the possibility of possible individual variations in sexual behavior and cause a distorting influence. Furthermore, very complete material must be available, in order to be able to pair freshly fertile females with overripe males, and overripe females with normally ripe males, and, finally, overripe females with overripe males. The material which I used in 1920 was not sufficient for these demands. Furthermore, I did not have the opportunity in the limited space available in the Munich Zoological Institute, to raise all the caterpillars of a given nest, but rather was forced to undertake a significant reduction in the count of individually rich nests. Nevertheless, I can report the following today.

First I would like to offer the results of five normal nests; they yielded roughly equal numbers of females and males. There was, except for Culture 2, an excess of females; this was so small, however, that it fell well within the margin of error.

1.	10 ♀	8 ♂
2.	16 ♀	19 ♂
3.	11 ♀	9 ♂
4.	10 ♀	10 ♂
5.	19 ♀	13 ♂
Total	66 ♀	59 ♂

Among the overmature cultures I show in the following list in the second column how many days the overmaturity consisted of. /291

1.	2 days	47 ♀	28 ♂
2.	4 days	34 ♀	22 ♂
3.	5 days	26 ♀	17 ♂
4.	5 days	39 ♀	11 ♂
5.	5 days	15 ♀	20 ♂
6.	6 days	7 ♀	
	Total	168 ♀	98 ♂

From this data it can certainly be said that overmaturity has no physical effect on the male sex as in frogs. Rather the opposite results would appear more likely. In total, the increase in females is very significant; it leads to a sexual difference of 55 (55 ♂ to 100 ♀). The odd mutation of females appears best in Culture 6 with six days overmaturity. The culture was very severely damaged by the overmaturity. From the total of 100 eggs contained in the nest, only 20 caterpillars emerged, of which 12 perished after leaving the coccoon or died shortly thereafter without eating. Of the 8 remaining animals, a severely crippled animal died somewhat later. The seven remaining animals grew slowly and were exclusively females. What is striking in this phenomenon are two points: 1. In the first culture, despite an overmaturity of only two days, the count of females was so significant; 2. The preponderance of males in the fifth culture, despite the overmaturity of five days. I cannot present explanations for both of these occurrences. I would only like to mention that on those days on which mating of the butterflies took place, a particularly high temperature prevailed which had an unfavorable influence on the development of the culture. I have therefore repeated this year the culture experiments with Lymantria on a much more complete set of material and under variable, very closely observed external conditions.

Even though the results of the butterfly experiments were so unexpected, /292
it is in my opinion not difficult to explain the results of the frog and butterfly experiments on a common interpretation. It first must be stated that in contrast to my previous interpretation and at variance with the situation in butterflies, the females in frogs are homogeneous, which has great relevance in the light of what was reported previously. It is possible, then, to offer the general rule that overmaturity favors the formation of the heterogametic sex, i. e. a change from the homogametic sex in favor of the heterogametic sex. It is apparently a question of involution or vitiation of the second factor determining sex in both cases, in the cases before us, of the X chromosome. In the simultaneous presence of two sex chromosomes the protoplasm of the ova is able to gain the ability by means of overmaturity to so influence the second sex chromosome that its effect is reduced or even involuted, a process which is perhaps appropriate to shed light on the development of morphological, sex-determining causation.

Regarding the previously-reported information, I would like to make two further points.

In my previous publications, I considered the possibility on the basis of theoretical considerations, that early maturity, i. e. premature advent of follicle disruption and premature transfer of the ova into the oviduct could bring about a sex-determining influence. Through my experiments of the last year, I have abandoned this assumption. I have had frog materials collected at the very beginning of restocking. At this time, I came upon five pairings in which the embrace of the male lasted three weeks without result. In two cases investigations yielded the result that the ovaries had degenerated. I think it is apparent that this degeneration was a result of a premature /293
embrace and a resultant disturbance of circulation, not the cause of the fruitless course of events. Certainly further experiments would be desirable in this case. If premature embrace has a damaging effect on the eggs, then an influence on sex determination from it is highly unlikely. In the same sense, another experience is noteworthy. In many embraces observed from their very beginning which later lead to oviposition, the duration was very variable, varying in general between 24 and 48 hours, apparently because the varying maturity was different in individual cases. In one case, the copula lasted 8 days before oviposition began. These differences in the ease noted in the follicle disruption and the transport of the eggs into the uterus, had no effect on the sex ratio.

The second point which I would like to discuss refers to the question of whether the material contained in the uterus of the female consists of an equivalent mixture of the different sexes in differentiated eggs or whether there is any form of sorting or layering. It would be conceivable that the female eggs leave the ovary first and therefore fill the lower segment of the uterus primarily, and that in contrast the male eggs take up the upper segment and thus are emptied last. Such a layering of eggs would not even be suggested in the case of homogametic females, and is moreover very unlikely anyway. For the sexual ratio of 50 ♂ : 50 ♀ is obtained regardless whether the pair spawns fully or only the first hundred eggs. In the latter case, the sex ratio of the first portion of eggs would have to be displaced toward the female direction. I have not tested this question to any degree of certainty. I cultured the material from the first oviposition as well as the last separately without interrupting spawning, thus without any influence of overmaturity. In both portions the sex ratio was completely equivalent. I have come to the conclusion, therefore, that /294 the only influence which can bring about a change in the sex-determining factors must operate on the eggs already in the uterus.

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